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CONSTRUCTION, EXPRESSION AND CHARACTERIZATION OF A MURINE/HUMAN CHIMERIC ANTIBODY WITH SPECIFICITY FOR HEPATITIS B SURFACE ANTIGEN

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Abstract—A murine/human chimeric antibody with specificity for Hepatitis B surface antigen has been produced by genetic engineering. The light and heavy chain variable region exons encoding the murine monoclonal antibody 2H1 were isolated and inserted into mammalian expression vectors containing the human kappa and gamma 1 constant region exons. The chimeric genes were transfected into murine Sp2/0 hybridoma cells by electroporation and transfectomas secreting chimeric antibody were isolated. Secretion levels ranged from 1–7 pg/cell/24 hr. The chimeric antibody bound specifically to Hepatitis B surface antigen and competed effectively with the parental murine monoclonal antibody for binding to these sites. Chimeric 2H1 is the first clinically relevant, genetically engineered anti-viral antibody and may represent an improved agent for the prevention of hepatitis B virus transmission.

INTRODUCTION

HBV infection is an important public health problem throughout the world affecting at least 200 million people (Bitter *et al.*, 1988). The major mode of transmission of HBV is from HbsAg positive pregnant woman to their offspring during the perinatal period (Hollinger *et al.*, 1985). In order to prevent perinatal infection, hepatitis B vaccine, in conjunction with anti-hepatitis B immunoglobulin, is administered to infants at birth and this treatment is highly effective (Beasly *et al.*, 1983). However, the currently available polyclonal antibody against HBsAg is derived from pooled human anti-HBsAg positive plasma. The potential risk from infectious agents and diminishing sources limit the clinical use of human plasma derived anti-hepatitis B immunoglobulin for passive immunization. An appropriate mAb against HBsAg would offer several advantages over the human polyclonal antibody. First, a mAb would pose no risk of infecting patients with AIDS, hepatitis, or any other infectious disease. Second, the supply would be virtually unlimited. Third, the titer of neutralizing antibody would be high and constant which would eliminate variability between lots. Finally, the high specific activity of monoclonal preparations would lead to lower therapeutic doses and decreased costs. Moreover, a mAb reactive with a single HBsAg epitope has been shown to be capable of effectively neutralizing the virus (Harada *et al.*, 1989).

Most mAbs are of murine origin and may have limited utility in human therapy because they usually elicit an immune response in patients (Miller *et al.*, 1983; Shawler *et al.*, 1985). Such a response reduces therapeutic efficacy and also may cause undesired clinical side effects. Human mAbs would circumvent this problem but they are difficult to produce and often have low affinity. Moreover, human hybridomas are usually unstable and secrete Ig at low levels (Carson and Freimark, 1986; Morrison, 1985). Chimeric antibodies consisting of murine V regions and human C regions represent a compromise. Conceptually, a chimeric antibody would retain the affinity and specificity of the parental murine mAb, and would eliminate the patient immune response to the murine C regions. Also, transfectomas producing chimeric antibodies appear to be stable and secrete antibody at levels sufficient for commerical application (Dorai and Moore, 1987; Beidler *et al.*, 1988). A number of chimeric antibodies have been produced recently (Morrison *et al.*, 1984; Sahagen *et al.*, 1986; Sun *et al.*, 1987) and some are entering clinical trials (LoBuglio *et al.*, 1988). Here, we report the production of a chimeric antibody with the affinity and specificity of 2H1, a murine mAb specific to HBsAg (Swenson *et al.*, 1987). Chimeric 2H1 may have the potential of replacing human polyclonal antibody in the prevention of vertical transmission of HBV during the perinatal period as well as in dialysis patients, transplant patients and others who are exposed to HBV.

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†Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; VH, variable heavy chain; VL, variable light chain; CDR, complementarity determining region; Ig, immunoglobulin; C, constant; V, variable; H, heavy; L, light; mAb, monoclonal antibody; D, diversity; J, joining; FCS, fetal calf serum.

MATERIALS AND METHODS

Cell culture. The 2H1 and Sp2/0-Ag14 hybridomas were cultured in RPMI-1640 media supplemented with 10% FCS and 2 mM L-glutamine. Transfectomas were grown in DMEM containing 5% NCTC-109, 10% FCS, 2 mM L-glutamine, 1 μg/ml mycophenolic acid, 50 μg/ml Xanthine and 500 μg/ml Geneticin (Gibco). All lines were maintained at 37°C in 7% CO_2.

Nucleotide sequencing. The nucleotide sequence of both the H and L chain V regions of 2H1 mRNA was determined by the primer extension method of Sanger *et al.* (1977) using AMV reverse transcriptase. Total RNA was extracted from 2H1 cells with guandinium thiocyanate (Chomczynski and Sacchi, 1987) and poly A^+ mRNA was isolated by oligo (dT) cellulose chromatography (Aviv and Leder, 1972). Universal primers, corresponding to the C regions of H and kappa L chains, were used in the initial sequencing (Kaartinen *et al.*, 1983). DNA sequencing of the cloned VH and VL regions was performed directly on pUC subclones using universal forward and reverse primers (Chen and Seaberg, 1985). Additional primers were synthesized on an Applied Biosystems Model 380A DNA synthesizer to complete the sequencing.

Southern analysis. Genomic DNA of 2H1 and Sp2/0-Ag14 cells were digested with multiple restriction endonucleases, fractionated by electrophoresis through 0.7% agarose gel, and transferred to nitrocellulose. Hybridization was carried out at 42°C overnight in Stark's buffer (Wahl *et al.*, 1979) containing 50% formamide and 1–10 ng/ml of a random oligodeoxynucleotide labelled probe (Feinberg and Vogelstein, 1983). The filters were washed twice in 0.1 × SSC, 0.1% SDS at 65°C for 30 min. Hybridization conditions for 5'-end-labelled V region specific oligonucleotide probes were as above except that the formamide was omitted and the filters were washed in 2 × SSC. The filters were then autoradiographed using Kodak XAR film and intensifying screens (Dupont) at −80°C.

Isolation of murine 2H1 VH and VL regions from a genomic library. A genomic DNA library from the 2H1 cell line was constructed in the λ phage vector EMBL-3. High molecular weight DNA was isolated, partially digested with restriction endonuclease Sau3A and fractionated by agarose gel electrophoresis (Maniatis *et al.*, 1982). DNA fragments between 9–23 kb were eluted onto a glass fiber filter, extracted and ethanol precipitated. The DNA was then ligated with λ EMBL-3 that had been digested with BamH1 and dephosphorylated. Recombinant λ phage were packaged with GIGAPACK GOLD extract (Stratagene) and plated at a density of 4.4×10^5/150 mm diameter petri dish. Duplicate filter lifts were prepared using nitrocellulose filters (Schleicher and Schuell). Filters were hybridized with random oligodeoxynucleotide labelled probes. The probe used for identifying the VH region was the 1.3 kb HindIII/PstI DNA fragment (HPH) containing murine J_{H4} and a portion of the intron between the V and C region of the H chain. The probe used for detecting the VL region was the 1.1 kb PstI/HindIII DNA fragment (HPL) derived from the murine L chain intron (see Fig. 1). Putative positive clones were isolated and purified by up to four rounds of rescreening.

Transfection of DNA into mouse cells by electroporation. DNA was introduced into murine hybridoma Sp2/0-Ag14 cells by electroporation. $1–2 \times 10^7$ actively growing Sp2/0-Ag14 cells were washed and resuspended in 1.0 ml of sterile PBS. Thirty micrograms of each chimeric, Igκ and IgG1, plasmid was added to the cell suspension. The DNA/cells were transferred to a pre-cooled shocking cuvette, incubated on ice at least 5 min and then a 0.5 kv/cm electric pulse was delivered for 10 msec (Transfector 300, BTX). After shocking, the DNA/cell mixture was returned to ice for 10 min and then diluted in 10 ml of DMEM containing 5% NCTC-109 and 10% FCS and incubated at room temperature for 10 min. Finally, the cells were transferred to a 37°C incubator with 7% CO_2 for 48 hr before plating in selective medium, containing 1 μg/ml mycophenolic acid and 50 μg/ml Xanthine. Cells were plated in 96-well plates at 3×10^4 cells/well.

ELISA analysis. Microtiter plates were coated with goat anti-human IgG antibody (Fc specific, Sigma) at 500 ng per well. Samples (100 μl per well) were incubated for 2 hr at 37°C with gentle shaking. After washing, 100 μl of horseradish peroxidase-conjugated goat anti-human kappa antibody (1:2500 v/v, Sigma) was added to each well and the plates were incubated at 37°C for 1 hr. The plates were washed again and 100 μl of citrate-PO_4 buffer (pH 5.0) containing 0.03% H_2O_2 and 0.04% *o*-phenylenediamine were added per well to produce a color change. The plate was developed for 30 min in the dark. The reaction was stopped by adding 50 μl of 4.5 *M* H_2SO_4, and the optical density was measured at 490 nm in a Microplate Reader (Molecular Devices). To determine the concentration of antibody produced by the transfectomas, purified human IgG (CALTAG Laboratory) was used to generate a standard curve.

HBsAg binding assay. Chimeric 2H1 antibodies were tested for their ability to bind HBsAg by using the AUSAB test (Abbott Laboratories), an RIA for the detection of anti-HBsAg antibody. Tissue culture supernatants (100 μl) from clones positive in the ELISA was added to wells containing HBsAg-coated beads. The beads were incubated at a room temperature for 18 hr, then washed twice with 5 ml of deionized water. Two hundred microliters of ^{125}I-labelled HBsAg (from human plasma) was added to the bottom of each reaction well and incubated at room temperature for 4 hr. After washing, the beads were transferred to assay tubes and counted in a gamma

counter (Picker Compac 120). Three positive and seven negative controls were included in each assay.

Western analysis. Purified recombinant HBsAg (kindly provided by Dr. R. Douglas) that was dissociated by boiling in 1% SDS—β-mercaptoethanol or undissociated by a similar treatment in the absence of β-mercaptoethanol (Hsiung *et al.*, 1984) was electrophoresed on a 10% SDS-polyacrylamide gel. After gel electrophoresis, the separated proteins were transferred to nitrocellulose by electroblotting at 1.0 amp for 30 min in 25 m*M* Tris (pH 8.3), 192 m*M* glycine, 20% methanol (V/V) buffer (Towbin *et al.*, 1979). Nitrocellulose containing separated proteins was incubated in 3% BSA, TBS (50 m*M* Tris, pH 8.0, 150 m*M* NaCl) at 37°C for 1 hr. The nitrocellulose was then incubated in 3% BSA-TBS containing a 1:5 dilution of 2H1 or chimeric 2H1 antibody (both at a final concentration of 8.4 μg/ml) at 4°C overnight. The nitrocellulose was washed four times with 3% BSA-TBS at room temperature for 30 min, then incubated with a 1:500 dilution of ^{125}I-anti-human IgG (Amersham) or ^{125}I-anti-mouse IgG (New England Nuclear) at room temperature for 2 hr. The blots were washed with TBS-0.05% Tween 20 for 30 min with three changes and exposed on Kodak film overnight. Duplicate gels were silver stained.

Biosynthetic labelling of 2H1. Ten million 2H1 cells were washed three times with methionine-free DMEM at room temperature and labelled for 20 hr in methionine-free DMEM containing 10% FCS and 100 μCi/ml ^{35}S-methionine (1094 Ci/mMole, New England Nuclear) (Thamanna and Scharff, 1983). The culture medium was harvested and used to label a new batch of 10^7 2H1 cells for another 20 hr. Labelled antibody in the culture supernatant was dialyzed against PBS and used for assays without further processing.

Competition immunoassays. Microtiter plates with removable wells (Dynatech) were coated with 15 ng HBsAg in 50 μl PBS per well at 4°C overnight and blocked with 1% BSA-PBS. After washing three times with PBS, a solution containing 20 μl of ^{35}S-2H1 and varying amounts of competing antigen or antibody was added to each well and incubated for 3 hr at room temperature. The plates were washed three times with PBS, then bound radioactivity was measured in a liquid scintillation counter. Competing HBsAg was tested over a range of 0.16–12.5 m*M*. The concentration of competing antigen that produces a 50% inhibition of 2H1 binding is referred to as the IC_{50} value. The antibody competition was carried out using unlabelled 2H1, chimeric 2H1 or a control antibody, Pc7.1 (anti-phosphocholine).

RESULTS

Restriction analysis and mRNA sequencing of the 2H1 V regions. Genomic DNA from 2H1 and its fusion partner, Sp2/0-Ag14, was digested with various restriction endonucleases and analyzed by Southern blots to identify the functionally rearranged V regions. This analysis showed that the heavy chain V region was contained in a 6.3 kb HindIII fragment and that the light chain V region was in a 4.2 kb HindIII fragment. Partial restriction enzyme maps (later confirmed and extended by DNA sequencing of the genomic clones) and a representative Southern blot are shown in Figs 1 and 2. In order to synthesize 2H1-specific oligomer probes for screening genomic clones, and to confirm that the correct genes had been cloned, 2H1 poly A$^+$ mRNA was sequenced. Nucleotide sequences of approximately 110–120 bases of the V regions of heavy and light chains were obtained from the initial sequencing reaction. Probes

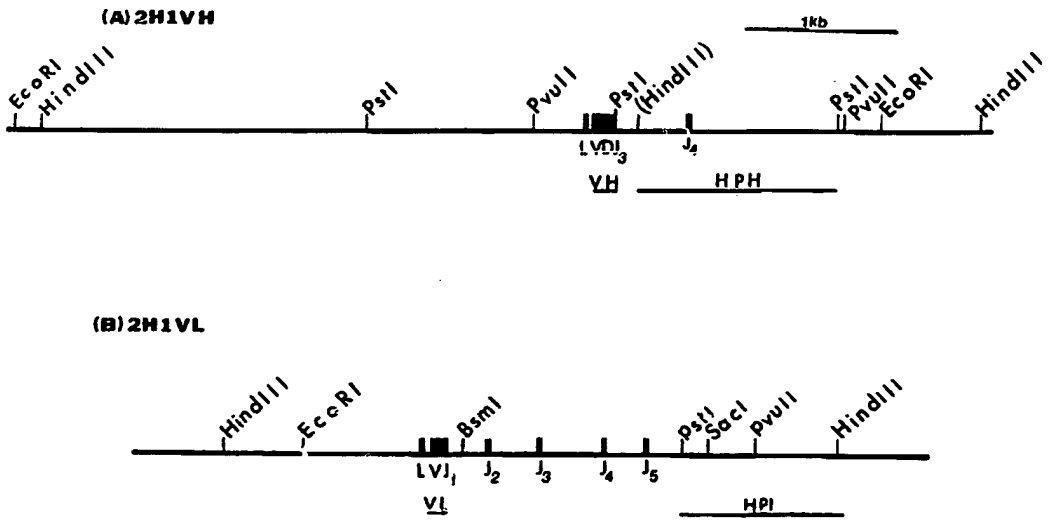

Fig. 1. Partial restriction map of 2H1 V region exons. Panel A shows a DNA fragment containing the 2H1 heavy chain VDJ_3 region. Panel B shows a DNA fragment containing the 2H1 light chain VJ_1 region. The locations of the probes used in this paper are indicated.

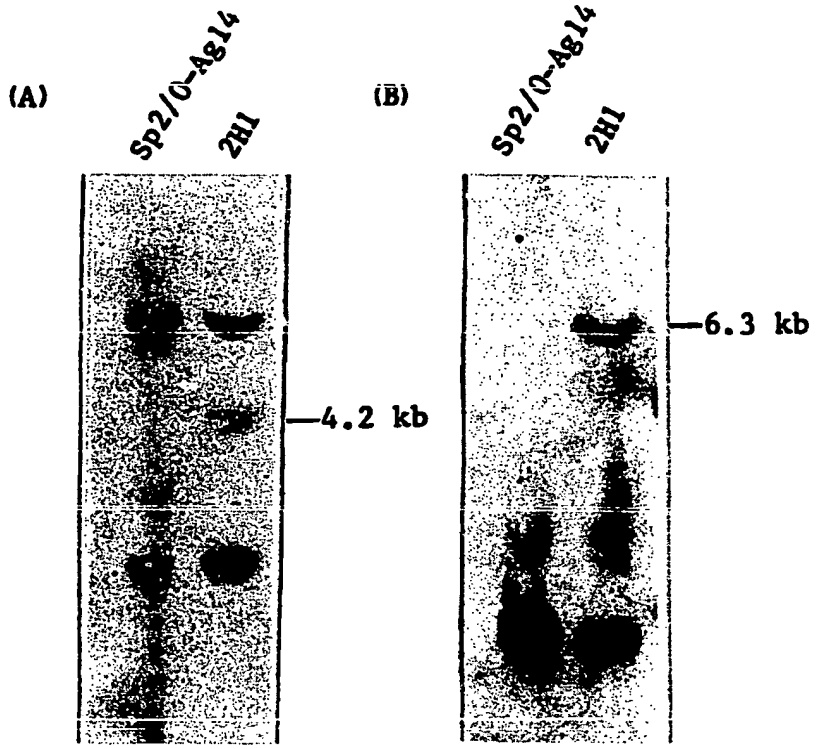

Fig. 2. Southern analysis of 2H1 and Sp2/0-Ag14 DNA. DNA was digested with HindIII and hybridized with the light chain probe, HPL (A) or the heavy chain probe, HPH (B). Panel A shows a unique 4.2 kb HindIII band containing the VL exon. Panel B shows a 6.3 kb HindIII band containing the VH exon. The sizes of the fragments were determined by comparison to HindIII digested λ phage DNA.

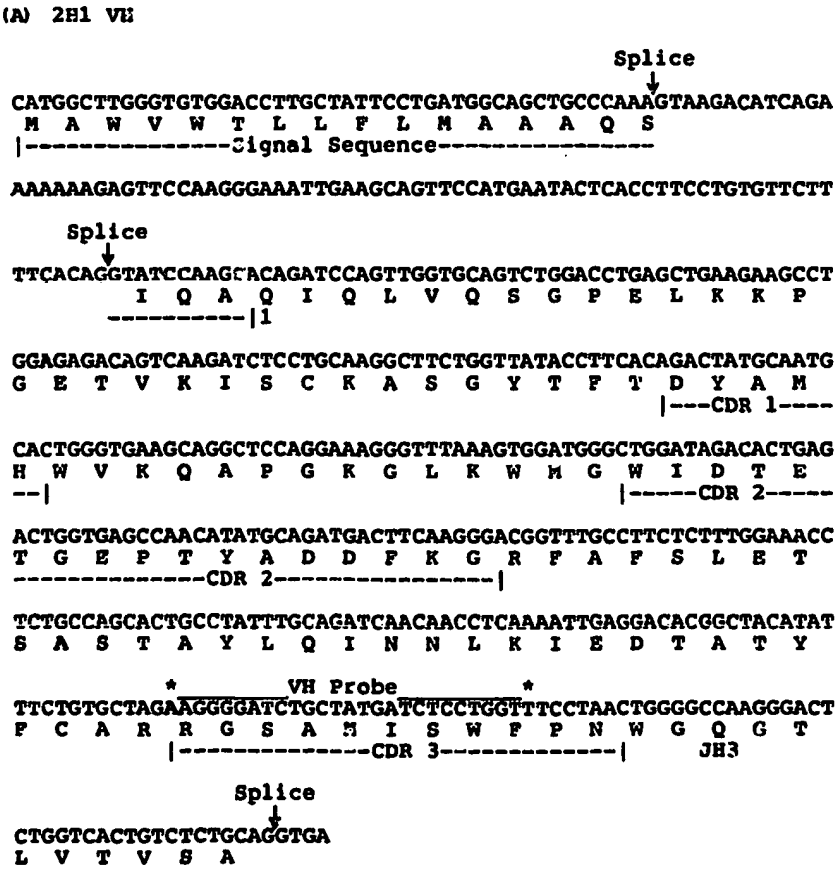

Fig. 3(A)

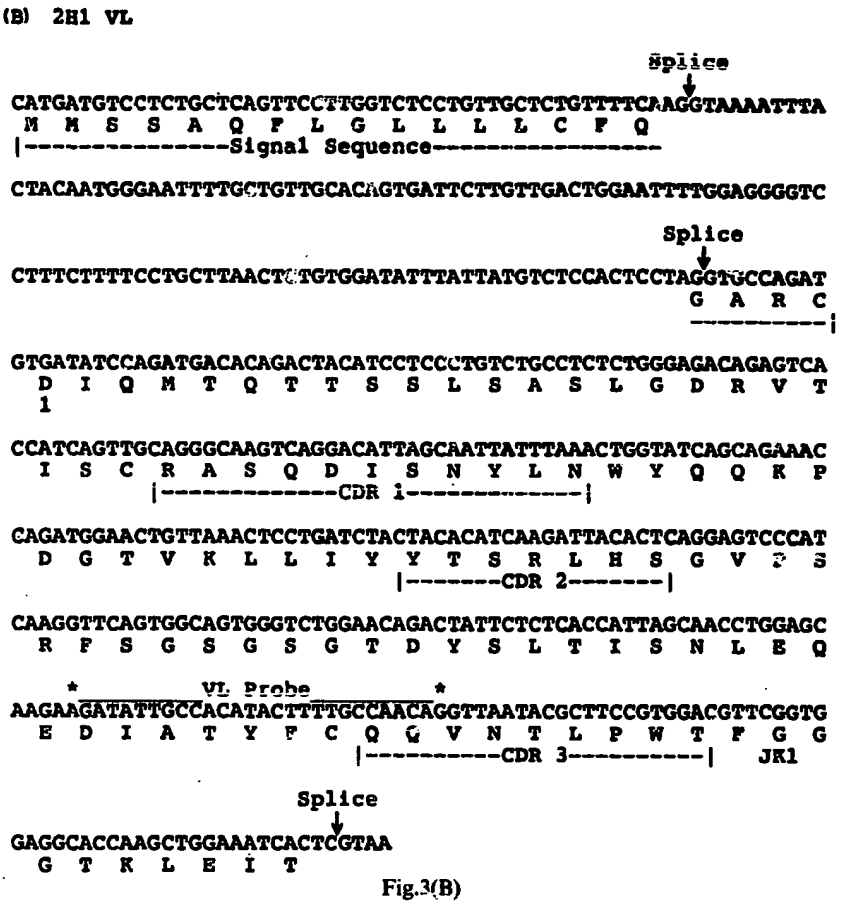

Fig.3(B)

Fig. 3. DNA sequence of the 2H1 VH and VL exons. The coding regions of 2H1 VH (A) and VL (B) are shown including the location of signal sequences, splice sites, CDRs, J segments and specific V region probes.

corresponding to CDR3 of both the L and H chains were synthesized and used to screen genomic libraries (see Fig. 3).

Isolation of VH and VL regions of 2H1. A genomic DNA library of 2H1 was prepared in λ-phage EMBL-3. The library, 3×10^6 phage, was screened initially with the fragment probes HPH and HPL. The 2H1-specific oligomer probes were used in subsequent rounds of rescreening to isolate pure plaques. Two H chain clones and three L chain clones were identified which hybridized strongly to both the fragment and 2H1-specific oligomer probes. Southern analysis showed that the H chain clones contained the expected 6.3 kb HindIII fragment as well as the 5.5 kb EcoRI fragment and that the L chain clones contained the 4.2 kb HindIII fragment (see Fig. 1). The 4.3 kb HindIII VL fragment was subcloned into pUC18 and the 5.5 kb EcoRI VH fragment was subcloned into pUC19.

DNA sequencing of the VH and VL regions of 2H1. The 1.6 kb PstI VH fragment and the 1.6 kb HindIII-BsmI VL fragment were subcloned into pUC19 and sequenced directly by the dideoxy method. The coding sequences are shown in Fig. 3. The positions of the signal sequences, splice sites, CDRs, and oligomer probes are indicated. Analysis of the sequences indicates that 2H1-VH is derived from the JH_3 minigene and is a member of the IIA subgroup (Kabat *et al.*, 1987). It was also found that the D segment in 2H1 is related, but not identical, to DSP 2.2. The sequencing also demonstrated that 2H1-VL is derived from the JK_1 minigene and belongs to the kappa V subgroup. It is interesting to note that the HindIII site located between JH_3 and JH_4 is missing from 2H1 due to a single base deletion. This causes the absence on Southern blots of the 2.3 kb HindIII fragment diagnostic of immunoglobulins using JH_3.

Construction and Expression of chimeric 2H1 H and L chain genes. The 5.5 kb EcoRI 2H1 VH fragment was cloned into the EcoRI site of a mammalian expression vector which contains the human gamma-1 C region and the *gpt* gene (Fig. 4). The 4.2 kb HindIII 2H1 VL fragment was cloned into the HindIII site of a similar vector which contains the human kappa C region and the *neo* gene (Fig. 4). The two plasmids were co-transfected into Sp2/0-Ag14, a non-Ig producing murine hybridoma, by electroporation. After transfection, the cells were grown for 48 hr in media without drug, and then transferred to media containing 1 μg/ml of mycophenolic acid and

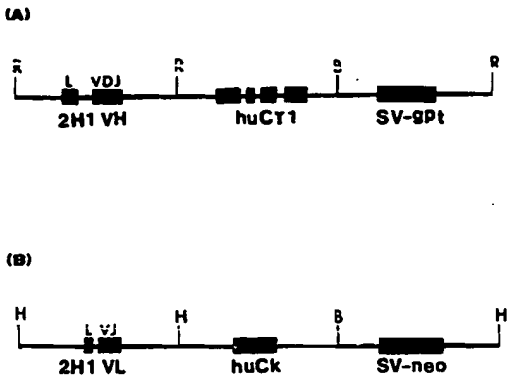

Fig. 4. Schematic diagrams of the chimeric murine/human heavy chain gene vector (A) and the chimeric light chain gene vector (B).

50 μg/ml Xanthine for initial selection. Transfection efficiency was approximately 1×10^{-5}. After two weeks, supernatants were assayed for the presence of chimeric antibodies, produced by the transfectomas.

Analysis of chimeric 2H1 transfectomas. Culture supernatants from cells resistant to mycophenolic acid were assayed for the presence of murine/human chimeric antibody by ELISA. The antibody used to coat the microtiter plates was goat anti-human IgG (Fc specific) and goat anti-human κ antibody conjugated to horseradish peroxidase was used to detect antigen-antibody complexes. The ten highest producing transfectomas were transferred to media containing both mycophenolic acid and geneticin (0.5 mg/ml) for selection of both transfected plasmids. Four of the clones survived this double selection and produced chimeric antibody at a rate of 1.2–7.0 pg/cell/24 hr (Table 1). None of the chimeric antibodies reacted with goat anti-mouse antibody.

Binding of chimeric 2H1 antibody to HBsAg. The ability of chimeric 2H1 antibody to bind HbsAg was tested in an RIA (Table 1). As shown in the Table, all four of the doubly selected clones were strongly reactive to HBsAg. Also, the RIA and the ELISA values of the samples were directly proportional. This was also true for the 24 hr expression level test.

The murine 2H1 antibody had been shown previously to recognize a conformational epitope on HBsAg (Swenson *et al.*, 1987). It binds undissociated, but not dissociated, HBsAg. The ability of chimeric 2H1 to distinguish between undissociated HBsAg was determined by Western analysis (Fig. 5). Purified HBsAg treated with or without β-mercaptoethanol was electrophoresed on SDS polyacrylamide gels, transferred to nitrocellulose and reacted with either 2H1 antibody or chimeric 2H1 antibody. As expected, chimeric 2H1 and 2H1 gave similar results. Neither antibody reacted with dissociated HBsAg, but both gave a signal with the undissociated antigen.

The relative affinities of chimeric 2H1 and 2H1 for HBsAg in a competitive binding test. Binding inhibition assays were performed to demonstrate that chimeric 2H1 antibody can compete effectively with the murine 2H1 antibody. As shown in Fig. 6, when 430 ng of unlabelled 2H1 was added to the reaction, the bound radioactivity decreased by 50%. At the same concentration of chimeric 2H1 the radioactivity was decreased by 49%. The inhibition curves of 2H1 and chimeric 2H1 are virtually identical. This result suggests that the chimeric 2H1 and 2H1 antibodies have the same affinity for binding to HBsAg. A competition RIA performed with biosynthetically labelled 2H1 and unlabelled HBsAg is shown in Fig. 7. The data demonstrate that 2H1 has a high affinity for HBsAg with an IC_{50} of 3.7 n*M*.

Table 1. Production level and HBsAg binding of chimeric 2H1

Transfectomas	Production level (pg/cell/24 hr)	Binding of HBsAg[a] (cpm)
B_3	7.0	20,654
D_9	3.7	20,500
E_6	3.5	17,897
A_2	1.2	15,374

[a]In the HBsAg binding assay, the cutoff value was 269 cpm. Samples with a cpm rate equal to or greater than 269 cpm would be considered reactive to HBsAg.

DISCUSSION

We have joined the DNA segments encoding the murine VH and VL exons from the 2H1 mAb specific for HBsAg to the DNA segments encoding human gamma-1 and kappa C regions. When the chimeric genes were introduced into non-Ig producing Sp2/0 cells, a functional chimeric antibody specific for HBsAg was assembled and secreted.

It has been reported that the major group and subtype specific antigenic determinants of the S region of the HBsAg are conformational-dependent and quite sensitive to reducing and denaturing conditions (Tiollais *et al.*, 1985). Cleavage of disulfide bonds within the envelope proteins of HBV results in a substantial decrease of binding of some monoclonal antibodies to intact HBsAg. As previously reported, murine 2H1 monoclonal antibody does not react with dissociated HBsAg (Swenson *et al.*, 1987). Our Western blot results showed that the chimeric 2H1 antibody was also non-reactive with dissociated HBsAg. From the results of the competitive binding assay, it was demonstrated that both chimeric 2H1 and 2H1 compete effectively with ^{35}S-2H1 for the antigenic sites on HBsAg. These results suggest that, although the chimeric 2H1 had been manipulated at the gene level, both 2H1 and chimeric 2H1 antibodies still recognize the same epitope and have a similar affinity for binding to HBsAg.

Liu *et al.* (1987) reported the construction of a murine-human chimeric heavy chain immunoglobulin derived from an anti-HBsAg hybridoma CRL 8017. Comparison of the VH region sequence of 2H1 with the VH region sequence of CRL 8017 reveals that both the heavy chains are derived from the JH_3 minigene, but the sequences are not identical.

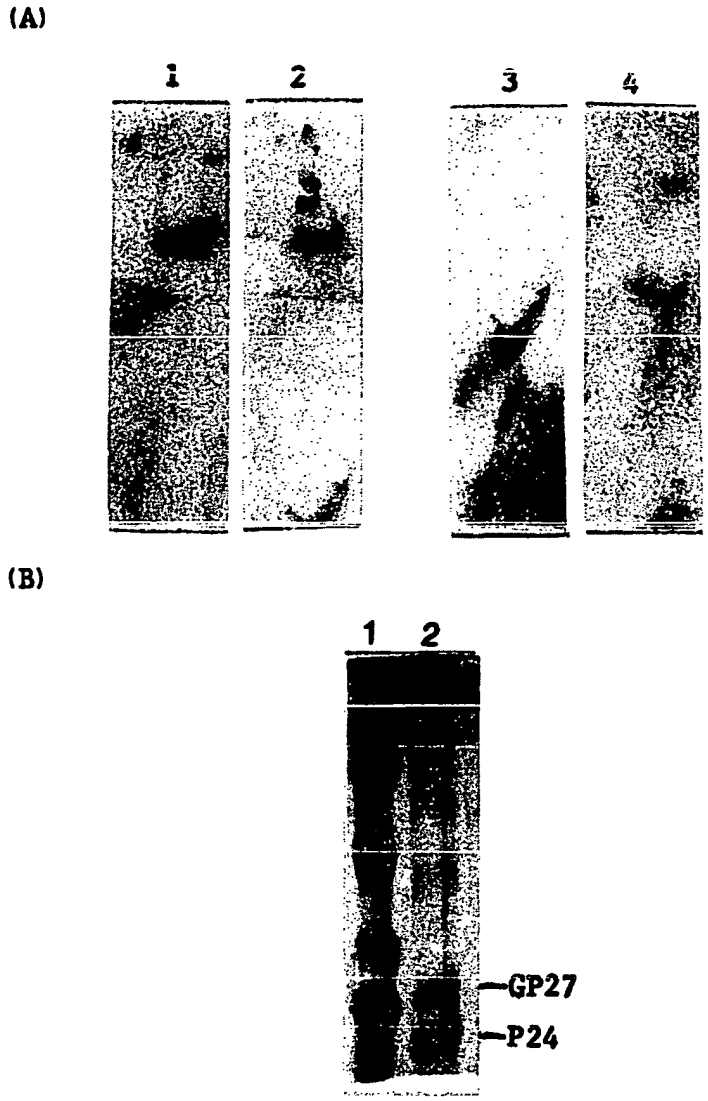

Fig. 5. Western analysis of 2H1 and chimeric 2H1 binding to HBsAg. Dissociated or undissociated HBsAg was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose as described in Materials and Methods. Panel A shows autoradiographs: Lane 1, undissociated HBsAg probed with chimeric 2H1; lane 2, undissociated HBsAg probed with murine 2H1; lane 3, dissociated HBsAg probed with chimeric 2H1; lane 4, dissociated HBsAg probed with murine 2H1. Panel B shows silver staining of dissociated HBsAg in a duplicate gel. Lane 1, molecular weight marker; lane 2, dissociated HBsAg. The position of the HBsAg subunits, GP27 and P24, are shown.

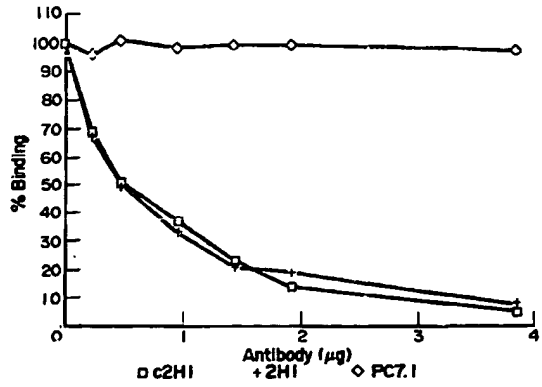

Fig. 6. Comparison of 2H1 and chimeric 2H1 in competition binding assays. Various concentrations of 2H1 (+), chimeric 2H1 (□) or an unrelated antibody, PC7.1 (◇) were used to compete the binding of ^{35}S-2H1 to HBsAg.

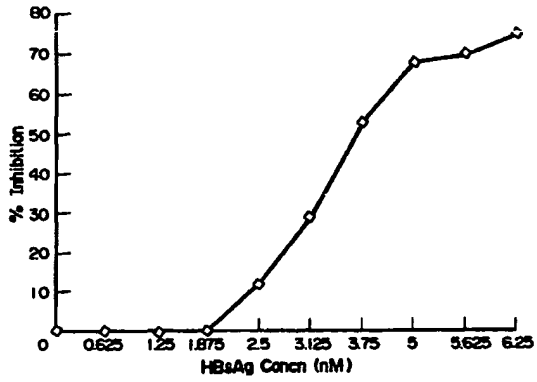

Fig. 7. Binding inhibition assay of 2H1 to HBsAg. Various concentrations of HBsAg were used to compete the binding of ^{35}S-2H1 to HBsAg.

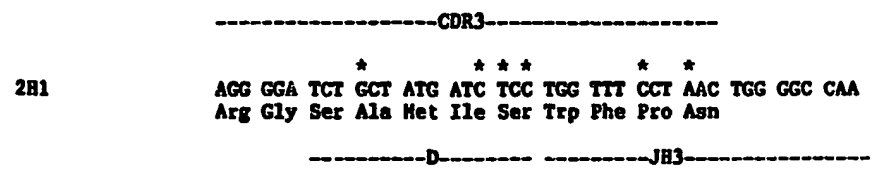

Fig. 8. Analysis of 2H1 CDR-3 region.

There are two single base changes in the 2H1 J region, and as a result, 2H1 differs from the germ line JH_3 at amino acid residues 108 and 109 (third and fourth codons of JH_3 minigene). Also, the D region in 2H1 is similar to the DSP 2.2 minigene, but not identical to it. The 2H1 D differs from the DSP 2.2 sequence by a single base substitution in the second codon of D (amino acid residue 102 of 2H1). In addition, there are three base substitutions in the codons for residues 104 and 105 of 2H1 that could have resulted from the diversity created by recombination and addition of N nucleotide residues at the D-J boundary. At the V-D junction also, there are six N nucleotide residues (Fig. 8). As a result, the CDR3 in 2H1 heavy chain is 11 amino acids long. In contrast, the CDR3 in CRL 8017 heavy chain is only 8 amino acids long and it corresponds to the germline sequence, with no apparent N residues present. Although it is unclear whether these antibodies recognize the same epitope on HBsAg, it is interesting that they are derived from the same D and J minigenes yet exhibit sequence differences possibly due to V-D-J recombination.

From the results presented, chimeric 2H1 may have the potential of replacing plasma derived human polyclonal antibody in the prevention of vertical transmission of HBV. The chimeric antibody retains the affinity and specificity of murine 2H1, but contains human C regions which should eliminate the immune response to the mouse C regions. Also, effector functions associated with the C regions should be enhanced when the chimeric antibody is used for immunotherapy. We are now in a position to test the efficacy of chimeric 2H1 in clinical trials.

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